ON THE DENATURATION OF CHROMOSOMAL DNA IN SITU*

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Denaturation studies on extracted DNA *in vitro* have made major contributions toward understanding the physical make-up of the molecule, its possible behavior in replication, and its response to various modifying agents, such as irradiation and incorporated base analogues. The present report deals with the effect of denaturing conditions on DNA in chromosomes prepared for microscopic observation, the visualization of the native and denatured states with the fluorochrome acridine orange, and preliminary consideration of the involvement of other chromosomal constituents in the process of denaturation.

Materials and Methods.—The tissues used in these investigations were larval salivary glands of *Drosophila melanogaster*. These were dissected in modified Ringer solution¹ and placed on gelatinized slides in a drop of 45 per cent acetic acid. In one experiment acid fixation was preceded by a 5-min treatment in 4 per cent neutral formaldehyde. The fixed glands were covered, squashed, and, after removal of the coverslip by freezing in liquid nitrogen, postfixed in ethanol-acetic acid (3:1), then passed through absolute and 95 per cent ethanol into 75 per cent ethanol for storage. Prior to subsequent treatments in aqueous solutions, the slides were hydrated gradually.

Enzymatic digestions were carried out over periods of 3 hr at 38°C. Desoxyribonuclease (Worthington), in 4×10^{-3} M MgSO₄, or ribonuclease (Worthington or N.B.C.) were used at a concentration of 0.3 mg/ml. The pH was adjusted to 7.0 with NaOH for DNase and Na₂HPO₄ for RNase. RNase solutions were freed of possible DNase activity by heating for 20 min in boiling water.

After preliminary treatments, slides were returned to distilled water, washed in acetate buffer (0.1 M, pH 4.5), and stained for 30 min in acridine orange (Harleco, 0.25 mg/ml in pH 4.5 acetate buffer). Before viewing, the preparations were washed several times in buffer, air-dried, covered with a fresh drop of buffer and a coverslip, and sealed with "Laktoseal" (Gurr). Observations of fluorescence patterns were made with Xenon illumination, a BG-12 exciter filter (4 mm, peak transmission at 4000 Å), and a Leitz "blue-absorbing" barrier filter.

Results and Interpretation.—The results obtained in terms of secondary fluorescence colors, are listed in Tables 1–3.

TABLE 1
SECONDARY FLUORESCENCE COLORS OF VARIOUS COMPONENTS OF DROSOPHILA SALIVARY GLAND CELLS STAINED WITH ACRIDINE ORANGE*

Treatment	Chromosomes	Nucleolus, cytoplasm
Stain alone	Yellow	Flame-red
Desoxyribonuclease	Barely visible	Pale flame-red
DNase buffer	Yellow-green	Pale flame-red
Ribonuclease	Yellow-green	Faint green
RNase buffer	Yellow-green	Pale flame-red

* In tissue fixed in 45% acetic acid.

Technical details of the treatments are given in the text. Color judgments are necessarily subjective. The assumption of their validity is based on independent examination of coded slides by both authors. In subsequent tables, distinctions between "yellow" and "yellow-green" or between different intensities of flame-red will not be made.

The assumption underlying these experiments is that denatured DNA can be distinguished from the native double-stranded molecule by its red fluorescence after acridine orange staining as opposed to the yellow-green color obtained in presumptively undenatured preparations. This expectation derives from the demonstration of red fluorescence in preparations of the single-stranded DNA virus ϕ X-174² and *in vitro* studies of various synthetic and natural polymers.³

Depurination of DNA by normal Feulgen hydrolysis (about 10 min in *N* HCl at 60°C) leads to a polymer where pairing between complementary bases is no longer possible. This treatment was used in a first attempt to observe the secondary

TABLE 2
SECONDARY FLUORESCENCE COLORS OF SALIVARY GLAND CHROMOSOMES STAINED WITH ACRIDINE ORANGE AFTER TREATMENT WITH HYDROCHLORIC ACID

Pretreatment	Acid Treatment			Fluorescence
	Conc.	Temp.	Time	
	(A) Fixed with 45% acetic acid			
None	1 <i>N</i>	60°C	15"-10'	Flame-red
	1 <i>N</i>	60°C	20'	None
	1 <i>N</i>	R.T.	5'	Flame-red
	0.3 <i>N</i>	R.T.	10'	Flame-red
	0.1 <i>N</i>	R.T.	10'	Yellow-green
	1 <i>N</i>	R.T.	5'	None
DNase	1 <i>N</i>	60°C	10'	Flame-red
RNase	3 <i>N</i> -0.01 <i>N</i>	R.T.	10'	Yellow-green
	(B) Fixed in 4% formaldehyde followed by 45% acetic acid			
None	1 <i>N</i>	60°C	10'	Yellow-green

fluorescence of nonhydrogen-bonded DNA. The expected result, red rather than yellow-green fluorescence, was obtained. (It is of interest to note that a similar effect on acridine orange staining was reported—without interpretation—by Subramaniam and Royan in 1960.⁴) The possibility that depurination *per se* rather than denaturation by hydrogen bond breakage is largely responsible for the change in secondary fluorescence can be ruled out: HCl treatments at concentrations and temperatures too low, and for times too short for appreciable purine removal,⁵ all produce a similar effect. We conclude that the red fluorescence in chromosomes after acridine orange staining indicates denaturation of the DNA by a change from a double- to a single-stranded state with or without appreciable depurination; such a change is known to occur in low pH solutions *in vitro*.⁶

Further reassurance of the validity of this conclusion is provided by a comparison of the effect of other treatments *in situ* with their known *in vitro* effects. With two points of divergence, the results are those which would be predicted from knowledge of the properties of DNA in solution. Thus, it appears that chromosomal DNA *in situ* is denatured by high temperature water⁷ and by a 90 per cent solution of formamide⁸ as would be expected. Moreover, unless formaldehyde is present before a heated preparation is allowed to cool, i.e., when the strands are separate, the double-stranded condition is re-established. (The action of formaldehyde in stabilizing single-stranded DNA is well known.⁹) It is possible that the maintenance of the denatured state by formaldehyde treatment may involve an effect of this agent on chromosomal protein, since fixation of freshly dissected tissues in formaldehyde prevents denaturation.

The apparent ease with which heat-denatured chromosomal DNA *in situ* undergoes renaturation is one of two differences in behavior of DNA *in situ* relative to

in vitro studies. Marmur and Doty¹⁰ found that DNA extracted from nonmicrobial cells does not renature readily upon cooling. The second point of apparent divergence is observed on ribonuclease-treated slides: heat or cold dilute acid treatment of tissue incubated with RNase does not result in the red DNA fluorescence expected. However, since this color is obtained after such slides are treated with hot HCl or cold dilute acid followed by heat, we conclude that the presence of RNase—rather than the absence of RNA—prevents denaturation. This is not an unlikely explanation in view of the demonstrated stabilizing effect of RNase on extracted DNA above its own denaturation temperature (60°C).¹¹

TABLE 3
SECONDARY FLUORESCENCE COLORS OF SALIVARY GLAND CHROMOSOMES STAINED WITH ACRIDINE ORANGE AFTER POSSIBLE DENATURATION PROCEDURES

Before heat	Treatment during heat*	After heat	Fluorescence
(A) Heating			
None	30' H ₂ O	Cooling†	Yellow-green
None	20' H ₂ O plus 10' 4% HCHO	Cooling†	Flame-red
10' 4% HCHO	30' H ₂ O	Cooling†	Yellow-green
None	30' H ₂ O	Cooling†	Yellow-green
DNase	20' H ₂ O plus 10' 4% HCHO	10' 4% HCHO	Yellow-green
RNase	As above	Cooling	None
RNase plus 10' 0.01 N HCl	As above	Cooling	Yellow-green
None	As above	Cooling	Flame-red
		Cooling plus RNase	Flame-red
(B) Formamide treatment			
30 min at room temperature in:			
100% HCONH ₂			Yellow-green
90% HCONH ₂ in H ₂ O			Yellow-green
90% HCONH ₂ in 0.02 M NaCl + 0.002 M Na Citrate			Yellow-green
90% HCONH ₂ in 40% HCHO solution			Flame-red
4% HCHO solution			Yellow-green

* Heat treatments carried out at 75 ± 3°C.

† Both rapid cooling, that is, immersion in distilled water at room temperature, and slow cooling, taking approximately 40 min to attain room temperature, were used in these experiments; the rate of cooling did not affect the results. Rapid cooling was used where no dagger is shown.

Note added in proof: The formaldehyde employed in the studies described was supplied by Portz and used, unless otherwise indicated, without buffers or neutralization. In view of the low pH (about 3) of this product, the formaldehyde stabilization experiments on heated chromosome preparations have since been repeated with neutralized (CaCO₃) formaldehyde, with and without citrate buffer, of the above as well as of two other manufacturers. The results obtained were identical to those described.

Discussion.—It is evident from the data presented that DNA in the chromosomes of *Drosophila* salivary gland nuclei responds to denaturing treatments largely as would be expected from studies on extracted molecules. It follows that the physical characteristics of the molecule are not dramatically different when it is part of a more complex structure. It is also evident that the behavior of DNA in the chromosome under denaturation conditions reflects the fact that it is an integral part of a more complex structure. The latter conclusion derives from two already mentioned observations: the readiness with which renaturation appears to occur during cooling in the absence of formaldehyde and the effect on denaturation of fixation of chromosomal protein. Both of these phenomena warrant some additional discussion.

The lack of appreciable renaturation in cooling of DNA extracted from non-microbial cells¹⁰ has been attributed to the low probability that complementary

strands will come into effective contact with each other. This difficulty should not arise in chromosomally fixed DNA, where retention of fine cytological detail even after heating to 85°C for 30 min suggests that DNA is never free to move very far from its original position during denaturation. In the *Drosophila* salivary gland nuclei in particular, there are large numbers of homologous strands available for renaturation in each polytene chromosome, even if the original pairing relationships are not precisely regained.

Experiments with other tissues suggest that renaturation probably occurs in nonpolytene chromosomes as well; this is a prerequisite for the yellow-green acridine orange fluorescence observed in acid-fixed, and hence temporarily denatured, materials of many kinds. In so far as the precursor incorporation in the presence of calf thymus polymerase noted in acid-fixed chromosome preparations is attributable to permanently denatured DNA,¹² our observations lead us to conclude that such permanent denaturation is too restricted in amount to be detected by our methods.

The influence of chromosomal protein on denaturation is evident from the effect of heat treatment with or without prior formaldehyde fixation. In the former case chromosomal histone would be less subject to loss from the preparation; it can be readily visualized how this protein, particularly after its own denaturation by formaldehyde, could interfere mechanically with DNA strand separation. (Moreover, the facts that RNase inhibits some denaturation effects and that acid treatment can reverse this inhibition add further support to the notion that the condition of chromosomal DNA is influenced by its protein environment.) It is of interest to recall in this connection that histone has demonstrable effects on RNA production¹³ as well as on DNA synthesis.¹⁴

Finally, if the nature of the interaction between DNA and acridine orange, giving either yellow-green or red fluorescence, is primarily a function of the orderliness or helical content of the DNA molecule,³ the data and considerations presented here may place a limit on the amount of distortion a DNA molecule must undergo to exhibit a change in the interaction. The apparent ease of renaturation as well as the absence of any microscopically detectable change in the DNA pattern of the chromosomes suggest that in so far as the individual DNA molecule has perceptible dimensions at this level of resolution, these dimensions are not appreciably changed by denaturation.

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¹ Ephrussi, B., and G. W. Beadle, *Am. Naturalist*, **70**, 218 (1936).

² Mayor, H. D., *Proc. Soc. Exptl. Biol. Med.*, **108**, 103 (1961).

³ Steiner, R. F., and R. F. Beers, *Polynucleotides* (Elsevier, 1961), chap. 10.

⁴ Subramaniam, M. K., and S. Royan, *Current Sci.*, **29**, 186 (1960).

⁵ Thomas, R., *Bull. Soc. Chim. Biol.*, **32**, 469 (1950).

⁶ Zimmer, Ch., and H. Venner, *J. Mol. Biol.*, **7**, 603 (1963).

⁷ Doty, P., J. Marmur, J. Eigner, and C. Schildkraut, these PROCEEDINGS, **46**, 461 (1960).

⁸ Marmur, J., and P. O. P. Ts'o, *Biochim. Biophys. Acta*, **51**, 32 (1961).

⁹ Thomas, C. A., Jr., and K. I. Berns, *J. Mol. Biol.*, **4**, 309 (1962).

¹⁰ Marmur, J., and P. Doty, *J. Mol. Biol.*, **3**, 585 (1961).

- ¹¹ Felsenfeld, G., G. Sandeen, and P. von Hippel, these PROCEEDINGS, **50**, 644 (1963).
¹² Bollum, F. J., *J. Cell. Comp. Physiol.*, **62** (Suppl. 1), 61 (1963); Von Borstel, R. C., D. M. Prescott, and F. J. Bollum, *J. Cell Biol.*, **19**, 72A (1963).
¹³ Huang, R. C., and J. Bonner, these PROCEEDINGS, **48**, 216 (1962); Allfrey, V. G., V. C. Littau, and A. E. Mirsky, these PROCEEDINGS, **49**, 414 (1963).
¹⁴ Baxill, G. W., and J. St. L. Philpot, *Biochim. Biophys. Acta*, **76**, 223 (1963).

NITROGEN-15 MAGNETIC RESONANCE SPECTROSCOPY, I. CHEMICAL SHIFTS*

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Except for the original determination¹ of nuclear moments, nitrogen magnetic resonance spectroscopy has been limited to the isotope of mass number 14. Although N¹⁴ is an abundant isotope, it possesses an electric quadrupole moment, which seriously broadens the resonances of nitrogen in all but the most symmetrical of environments.² Consequently, nitrogen n.m.r. spectroscopy has seen only limited use in the determination of organic structure. It might be expected that N¹⁵, which has a spin of 1/2 and no quadrupole moment, would be very useful, but the low natural abundance (0.36%) and the inherently low signal intensity (1.04×10^{-3} that of H¹ at constant field) have thus far precluded utilization of N¹⁵ in n.m.r. spectroscopy.³

Resonance signals from N¹⁵ have now been obtained from a series of N¹⁵-enriched (30–99%) compounds with a Varian model 4300B spectrometer operated at 6.08 Mc/sec and 14,100 gauss. The samples were contained in 10-mm tubes and were spun for best resolution. The power levels were limited by the rather long relaxation times of N¹⁵; however, the signals could be readily observed in the absorption mode. Chemical shifts (Table 1) were measured by the sideband technique with respect to a sample of 8.57 *M* nitric acid which contained a trace of ferric chloride, but are reported with respect to anhydrous ammonia which is a more reproducible standard. Calibration was made either by the substitution method, or by placing the standard in a 5-mm tube concentric to the sample tube. Under conditions of optimum resolution (1 cps), a well-developed ring-out was observed from the resonance of the standard.

Table 1 shows the N¹⁵ chemical shifts measured so far. As expected, there is general agreement with shifts from N¹⁴ magnetic resonance studies^{1–4} of predominantly inorganic compounds.

For interpretation, chemical shifts are usually divided into diamagnetic and paramagnetic contributions.⁵ Diamagnetic shielding is taken to arise from rotation of the electron cloud around the nucleus at the Larmor angular velocity, whereas paramagnetic effects are taken to arise from deviations from idealized symmetry, i.e., increases of orbital angular momentum. Holder and Klein^{3c} and Schmidt, Brown, and Williams⁴ have interpreted N¹⁴ chemical shifts in this manner. The